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A Fluorescence Resonance Energy Transfer (FRET)-based Redox Sensor Reveals Physiological Role of Thioredoxin in the Yeast *Saccharomyces cerevisiae*

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Abstract

The physiological roles of the thioredoxin isozymes in the yeast *Saccharomyces cerevisiae* were investigated using a novel FRET-based redox probe, Redoxfluor. After establishing responsiveness of the probe toward thioredoxin, we followed the fluorescence signal of Redoxfluor expressed in the yeast and found that one of the thioredoxin isozymes, Trx2, was required for maintaining the redox status when stationary culture of the organism was exposed to starvation and mild-heat stresses. The failure to maintain redox balance under the tested condition preceded decreased viability of the *trx2* mutants, indicating the functional importance of the cytoplasmic thioredoxin in adaptation to environmental changes.

Keywords

FRET; redox regulation; thioredoxin; yeast physiology

Highlights

Redoxfluor indicated reduced state *in vitro* by thioredoxin application.

Starvation and mild-heat stresses caused oxidized cytoplasmic state of *trx2* mutants.

The oxidized state in the stress condition preceded viability loss of *trx2* mutants.

1. Introduction

Maintenance of intracellular redox status is an important cellular activity, whose failure is associated with diverse pathological and aging processes [1,2]. To prevent undesirable accumulation of reactive oxygen species (ROS), eukaryotic cells possess diverse antioxidant enzymes such as catalase, superoxide dismutase, and peroxidases [3].

The reducing equivalents required for these enzymes are mainly derived from glutathione or thioredoxin. Glutathione, which is present at millimolar levels inside cells, supports a broad substrate spectrum of peroxidase and reductase activities [4]. Thioredoxins are also utilized as electron donors for a subset of antioxidant enzymes, especially for the peroxiredoxin family [5]. The yeast *S. cerevisiae* has two cytoplasmic thioredoxins, namely Trx1 and Trx2, and the transcriptional activity of the latter is induced by multiple environmental factors such as oxidative stress and growth state transition to the stationary phase [6,7]. The oxidized forms of the cytoplasmic thioredoxins are reduced back by a thioredoxin reductase TrxR1 at the expense of NADPH.

To date, functional analyses of the yeast thioredoxins have been conducted mainly in the context of resistance to externally or experimentally added oxidative or reducing chemicals [6,8], and little is known about the physiological contributions of the isozymes to other environmental conditions. Moreover, while double deletion of the cytoplasmic thioredoxin genes was reported to cause growth defects and severe sensitivity to chemical applications [9], no clear phenotype resulting from the loss of a single thioredoxin gene has been reported, except for the reduced resistance to hydrogen peroxide caused by deletion of TRX2 [7]. This is partly because of the lack of appropriate methods to monitor intracellular redox status reflecting the redox balance of the electron donor molecules as well as the ROS accumulation.

In a previous study, we devised a new redox sensor protein named Redoxfluor [10] by utilizing the redox-sensing region of Yap1. *S. cerevisiae* Yap1 is one of the major transcription factors that sense intracellular redox status [11-13]. Redoxfluor is comprised of a tandem repeat of the Yap1C-terminal region flanked by two fluorescent proteins, Cerulean and mCitrine. Changes in FRET efficiency between

the two fluorescent proteins occur in response to various oxidative and reductive stimuli, indicating higher FRET rates during exposure to more reduced environments. This tendency contrasts with another FRET-based redox sensor protein [14] which shows lower FRET efficiencies under more reduced conditions. Notably, both of the FRET-based redox sensors also respond to changes in glutathione redox states *in vitro*, and thus can be used as sensors of the physiological redox status *in vivo* [10,15]. However, the response of Redoxfluor to another important redox compound, thioredoxin, remained to be investigated.

In this study we showed that Redoxfluor senses reducing activity of thioredoxin at physiological levels. By using this probe we examined impacts of the cytoplasmic thioredoxin gene disruptions on the redox status *in vivo*, which led to the discovery that TRX2 is necessary for maintaining the cytoplasmic redox status in response to environmental stresses induced by transfer of the cells to water at 37°C. Since the oxidized status observed in the mutant strain correlated with subsequent loss of viability, the physiological function of Trx2 in redox maintenance was elucidated.

2. Materials and Methods

2.1. Strains and culture conditions

All of the strains used in this study were derived from W303-1B as listed in Table I. For target gene disruptions, *Schizosaccharomyces pombe* HIS5, *Candida glabrata* LEU2 and TRP1 cassettes were amplified by PCR along with 40 bp extensions homologous to the upstream and downstream regions of the target genes (for the latter two cassettes using BYP1419 and BYP1805 plasmids, provided by National BioResource Project-Yeast, Japan, as templates), and introduced into the parental strain (YMO300). The strains used for microscopic and viability analyses (YMO301 to 308) were converted to prototrophs. Strains were cultured at 30° C in YPD medium [2% Bacto-Peptone (BD-Difco), 1% Yeast Extract (BD-Difco), and 2% D -glucose]. To attain stable and uniform expression of Redoxfluor, we carried out genome-integration of its construct into URA3 locus. To obtain cells in the exponential growth phase, overnight cultures in YPD medium were diluted in fresh YPD medium with an A_{610} of 0.3, and cultured at 30° C until the A_{610} of the culture reached 2.0. Stationary phase cultures were obtained by culturing in YPD medium at 30°C for 96 h, when the A_{610} of the culture exceeds 25.

2.2. Redoxfluor experiments *in vitro*

Redoxfluor protein was expressed and purified, and all of the biochemical experiments were conducted under anaerobic conditions as described in the previous study [10]. The purified protein was diluted to 0.5 mg/ml in Reaction Buffer (50 mM Hepes-NaOH, pH 7.0, 0.14 M potassium chloride, and 1 mM EDTA), incubated with 1 mM 4'-4'-dipyridyl sulfide (DPS) solution at 37°C for 10 min, and subjected to gel filtration on a PD-10 column (GE Healthcare). In order to prepare thiol-modified thioredoxin, recombinant Trx2 protein in Reaction Buffer was incubated with 1mM DTT at 37°C for 30 min, followed by addition of 10 mM iodoacetamide (IAA), and further incubated at 37°C for 2 hrs, followed by gel filtration on a PD-10 column to remove free IAA. The reaction was initiated by mixing 0.15 μ M of the DPS-oxidized Redoxfluor, 0.4 mM NADPH, 1 μ M of purified recombinant thioredoxin reductase (Trr1), 1 μ M of purified recombinant thioredoxin peroxidase (Tsa1), and the indicated concentrations of Trx2 in

reaction buffer. After incubation of the reaction samples at 30°C for the indicated periods, emission spectra of the reaction samples were obtained by excitation at 405 nm wavelength with RF5300PC spectrofluorometer (Shimadzu, Japan). The ratio of acceptor/donor fluorescence intensity $[I(\text{acc})/I(\text{don})]$ was calculated as summation of fluorescence emission intensities in the wavelength range from 524 to 526 nm (after subtraction of the corresponding background intensity values) divided by sum value in the range from 474 to 476 nm (after background subtraction). Cross-linking experiments with free thiol residues inside Redoxfluor proteins to methoxy-poly (ethylene glycol)-maleimide (mPEG-maleimide, from Laysan Bio) were carried out as described previously [10].

2.3. Visualization of Redoxfluor signal ratio by fluorescence microscopy

Ratio of fluorescence intensities from Redoxfluor in the *S. cerevisiae* strains was visualized with modifications of the method described previously [10]. We used an Olympus IX-70 inverted microscope equipped with a UplanApo 40x objective (Olympus) and a CoolSNAP HQ2 CCD camera (Photometrics). All of the filters for the fluorescence acquisition were from Omega: XF1201 (436AF8) for the excitation filter, XF2034 (455DRLP) for the dichroic filter, XF3075 (480AF30) for the emission filter passing through fluorescence from Cerulean, and XF3079 (535AF26) emission filter for fluorescence from mCitrine. The exposure time for acquiring the image was set to 0.1 sec with Uniblitz shutter system (Vincent Associates). In order to acquire the images through the filters for Cerulean and mCitrine fluorescence in the same field with a minimal time interval, the emission filters were mechanically exchanged in a filter wheel controlled with MAC5000 (Ludl Electronic Products). The images gained through the filters for Cerulean and mCitrine fluorescence were subjected to Ratio Image program in MetaMorph software (version 7.0, Universal Imaging).

2.4. *In vivo* assessment of signal ratio from Redoxfluor

In the images obtained through the filter for Cerulean fluorescence, more than 30 points were selected (1 point/ cell) as regions of interest (ROIs), each of which was located inside the cytoplasmic region with 5x5 pixel dimensions. The ROIs of the same position were allocated in the corresponding image through

the filter for mCitrine fluorescence. The signal intensities of the ROIs were measured with the Region Measurement program in the MetaMorph software. After subtraction of values from blank ROIs (outside the cell region), the signal intensities of the ROIs in the images through the filter for mCitrine were divided by the corresponding intensities of those through the filter for Cerulean (from which blank ROIs had also been subtracted). Three independent experiments were done for the data acquisition. The calculated values were averaged and represented as fluorescence intensity ratio (FI ratio) of Redoxfluor.

2.5. Determination of glutathione levels by LC-MS

The yeast cells equivalent to 5 OD₆₁₀ units were collected and resuspended in 1 mL of pure methanol pre-chilled at -80°C. The suspension was transferred to liquid nitrogen and subsequently thawed on ice. After centrifugation at 20,000 xg for 5 min, the supernatant fraction was retrieved and the pellet fraction was again resuspended in 1 mL of the pre-chilled methanol, subjected to the freeze and thawing and centrifuged to obtain the supernatant fraction. These supernatant fractions were combined and freeze-dried, and re-suspended in 0.5 mL of 1% acetonitrile solution. The samples were applied to Prominence nano HPLC (Shimadzu) - 4000QTRAP Mass spectrograph system (AB SCIEX) equipped with Hydrosphere C18 column (YMC) to determine reduced and oxidized glutathione levels as well as the amount of leucine as an internal control. The ΔE_{GSH} values representing the difference in redox potential of glutathione between wild type and mutant strains are calculated as below:

$$\Delta E_{\text{GSH}} = -RT/2F \ln(\text{Ratio of GSH content in the extract from a given mutant relative to that of wild-type strain})^2 / (\text{Ratio of GSSG content in the extract from the mutant relative to that of the wild-type strain})$$

R, Gas constant; T, absolute temperature (K); F, Faraday constant.

2.6. Viability assay

Cells in the stationary phase were collected and resuspended in water at 1 A₆₁₀ concentration, and incubated aerobically at 37°C. An aliquot of the sample was collected every 4 days and diluted 10,000-fold with fresh water. The diluted suspension (100 µl) was plated onto a YPD plate, and incubated at 30°C for 2-3 days before counting the number of colonies.

3. Results

3.1. Redoxfluor has increased its ratio of acceptor/donor fluorescence intensity in the presence of functional thioredoxin

The response of Redoxfluor to the reductive activity of thioredoxin was examined *in vitro* (Fig. 1). Pioneering studies on the molecular mechanisms of *S. cerevisiae* Yap1 and its *Schizosaccharomyces pombe* orthologue Pap1 activities demonstrated that the disulfide bond formations at their cysteine residues are mediated by thiol peroxidases (Orp1 and Tsa1 in *S. cerevisiae* and Tpx1 in *S. pombe*) [16-18], while cleavage of the bond is catalyzed by thioredoxin [19]. Based on these findings, the ratio of acceptor/donor fluorescence intensity [I(acc)/I(don)] of Redoxfluor was periodically determined after the probe protein was incubated with recombinant yeast thioredoxin (Trx2) in the reaction mixture of Tsa1 and thioredoxin regeneration system. The incubation saw a gradual increase in I(acc)/I(don), which indicated a shift to more reduced status of Redoxfluor (Fig. 1A). Since this increase in the ratio was suppressed in the absence of thioredoxin, we reasoned that Redoxfluor was reduced by an electron transfer from Trx2.

Based on data from high-throughput determinations of yeast protein concentrations [20], the intra-cellular concentration of the cytoplasmic thioredoxins (Trx1 and Trx2) was estimated to sub-micromolar level, given that the volume of a yeast haploid cell is $70 \mu\text{m}^3$. We examined whether Redoxfluor responds to Trx2 at this physiological level. As a result, the ratio [I(acc)/I(don)] was found elevated with increasing concentrations of Trx2 (Fig. 1B). Reduction of disulfide bonds in Redoxfluor by Trx2 was biochemically confirmed by subsequent incubation of the reaction mixture with a thiol-modifying reagent of 5-KDa size, mPEG-maleimide (Fig. 1C). Immunoblot analysis of the probe protein after the reaction showed that Trx2 application increased the highly modified population of the probe protein, reflecting increased thiol residues available for mPEG-maleimide modification. The Trx2 action on Redoxfluor was thiol-dependent, since the Trx2 derivative whose thiol residues had been

masked with iodoacetamide (IAA) did not increase in I(acc)/I(don) of Redoxfluor (Fig. 1B), nor augment the mPEG-maleimide modification (Fig. 1C)

Our previous study had shown that Redoxfluor sensed the redox states of glutathione couples (GSH-GSSG) [10]. We then examined whether the probe protein response with thioredoxin was also detected in the presence of a physiological level of glutathione couple. Notably, I(acc)/I(don) rose with increasing concentrations of thioredoxin even in the presence of the glutathione redox couple (Fig. 1B). These *in vitro* data strongly suggest that Redoxfluor is capable of sensing the reducing activity of thioredoxin under physiological conditions.

3.2 Loss of cytoplasmic thioredoxins caused no indication of oxidative shift by Redoxfluor under authentic culture conditions

Next we examined whether loss of cytoplasmic thioredoxins altered the redox status in the yeast *S. cerevisiae*, using strains expressing Redoxfluor under TDH3 promoter regulation. The introduction of Redoxfluor did not affect the cell growth of each strain (data not shown). The wild-type and mutant strains lacking one or both of the cytoplasmic thioredoxins (Trx1 and Trx2) were cultured in glucose (YPD) medium to exponential or stationary phase, and subjected to fluorescence microscopy for assessing fluorescence intensity ratios (FI ratios) of Redoxfluor which reflect the FRET efficiencies of the probe protein (Fig. 2). The image reconstituted from Redoxfluor fluorescence covered a large part of the whole cells except the vacuoles, which are represented as black or green circles, due to the absence of the intact probe protein in these organelles (Fig. 2A). Compared with exponentially growing cells, the cells in the stationary phase exhibited higher FI ratio in the cytoplasm, indicating a more reduced status in the stationary phase (Fig. 2A and 2B), which is consistent with metabolite profiles shown in a previous study [21]. No remarkable difference in the FI ratio of Redoxfluor was detected among the tested strains, regardless of the growth phase (Fig. 2A and 2B), suggesting that loss of the cytoplasmic thioredoxins did not affect the overall redox status under these culture conditions.

3.3 Loss of Trx2 leads to a more oxidized status of cells during incubation in water at 37°C

We hypothesized that the physiological function of the cytoplasmic thioredoxins might be to maintain redox status under environmentally stressful conditions. In order to test this notion, we transferred the stationary cultures of the strains expressing Redoxfluor to water, which gives rise to nutrient starvation, and/or incubation at 37°C (mild heat stress), both of which are plausible challenges to yeast cells in natural environments. Especially, heat stress is known to affect yeast intracellular pH [22], which is likely to influence the redox states of thiol moieties inside cells.

Microscopic assessment indicated that the cytoplasmic thioredoxin-null (*trx1Δtrx2Δ*) mutant underwent a marked drop in the FI ratio as a sign of oxidative redox status, when stationary phase cells were incubated at 37°C for 4 days, regardless of the incubation medium (Fig. 3A and 3B). Deletion of TRX1 alone caused a slight, not visually discernible decrease in the FI ratio when cells were transferred to water at 30°C or exposed to mild heat stress alone, and led to even higher FI ratio than the wild-type strain when treated with the combination of the stresses (Fig. 3A and 3B). In contrast, loss of TRX2 led to a remarkable decrease in FI ratio of Redoxfluor when the cells were transferred to water at 37°C (Fig. 3A and 3B). In parallel with this observation, deletion of TRX2 significantly decreased glutathione levels in the cells transferred to water at 37°C, which eventually gave rise to more oxidized state of glutathione in the TRX2-deleted strains (Fig. 3C). These data indicate that redox maintenance is impaired in the thioredoxin mutants under these stress conditions.

3.4 The oxidized status in the TRX2-deletion strain precedes a decrease in its viability

We next examined whether the observed oxidative status in the TRX2-deleted strain was associated with a physiological phenotype. For these experiments we followed the viabilities of the strains used for the microscopic study during prolonged incubation in water at 37°C. The initial 4-day incubation under the stress conditions did not cause a significant decrease in the viabilities of the wild-type or the single

TRX-deletion strains, but caused a severe loss of viability of the cytoplasmic thioredoxin-null (*trx1Δtrx2Δ*) mutant (Fig. 3C). Importantly, further incubation up to 12 days resulted in a clear viability decrease in the TRX2-deletion strain than in the wild-type or TRX1-deletion strain (Fig. 4). These data together with the microscopic result strongly suggests that Trx2 function in the redox maintenance may contribute to supporting cell survival under the environmentally stressful conditions.

4. Discussion

Biochemical analysis (Fig. 1) demonstrated the response of Redoxfluor to the reductive activity of the yeast thioredoxin Trx2. This response was also detected without addition of the peroxiredoxin (Tsa1) in the reaction mixture, but with much slower kinetics and to a lesser extent (data not shown). This implies that the reduction process of Redoxfluor is facilitated by Tsa1. A plausible explanation of the Tsa1 role in the Redoxfluor response is that Tsa1 transiently forms a disulfide bond with Redoxfluor and the electron transfer from thioredoxin to the probe protein is mediated by the bound Tsa1 molecule, in analogy to the involvement of the peroxiredoxins in the regulations of Yap1 and Pap1 activities [16-18],

The responsiveness of Redoxfluor toward thioredoxin, in combination with other previously-reported characteristics of this probe protein [10], enables us to visualize integrated redox status encompassing the redox states of both glutathione and thioredoxin as well as intra-cellular ROS levels. Although another redox sensor protein, roGFP2, was reported to have little response toward human Trx in the minute time scale [23,24], it is possible that the steady-state signal gained from the probe protein may also be affected by thioredoxin.

Cells under normal culture conditions did not show remarkable differences in FI ratio of Redoxfluor depending on the presence of the TRX genes (Fig. 2), although transcriptional induction of TRX2 in the stationary phase was reported previously [7]. In these situations, activities of other redox-related enzymes such as glutathione dependent peroxidases, glutaredoxins, and catalases may be sufficient to maintain the redox status, making the effect of TRX gene disruptions undetectable. In a previous study, double deletion of TRX1 and TRX2 was found to cause constitutive activation of Yap1 activity, leading to induction of genes encoding redox-related enzymes [9]. Such a feedback system may be responsible for the redox maintenance in the absence of cytoplasmic thioredoxins.

The mutant strains lacking either of the cytoplasmic thioredoxins exhibited distinct indications of redox status from Redoxfluor, when the cells were exposed to both starvation and mild-heat stresses (in water at 37°C): while the TRX2-deleted strain showed more oxidized status than the wild-type strain, the

TRX1-deleted mutant showed more reduced status (Fig.3). One plausible hypothesis for explaining this result is that TRX1 deletion may up-regulate expressions of TRX2 or other anti-oxidant enzymes. Future studies addressing interrelationship between thioredoxin expressions and those of other redox-related enzymes will be necessary for the overall understanding of redox regulation.

In this study we aimed to reveal the function of thioredoxin in a physiological context, and chose to expose the cells to water and/or mild heat stress. Incubation of cells in water is a widely-used method to determine yeast chronological life span [25], and a previous study included the temperature shift to 37°C in the life-span assay to accelerate the decline in cell viability [26]. Thus, based on the data of this study (Fig. 4), Trx2 could be defined as a positive factor for extending longevity of the yeast. This notion is consistent with recent findings that peroxiredoxin proteins (acceptors of electron from thioredoxin) are important for establishing longevity in several experimental systems [27], and hence the physiological importance of thioredoxin will be unveiled to a greater extent in relation to longevity.

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FIGURE LEGENDS

FIGURE 1. Redoxfluor response to thioredoxin *in vitro*. **A.** Time course of change in the ratio of acceptor/donor fluorescence intensity of Redoxfluor after addition of thioredoxin. Purified and pre-oxidized Redoxfluor protein in a reaction mixture (See Materials and Methods) was incubated with (closed circle) or without (open circle) 5 μ M of recombinant Trx2 at 30°C for the indicated time periods. The ratio value $[I(\text{acc})/I(\text{don})]$ was calculated as described in Materials and Methods. The error bars indicate standard deviations from 3 independent experiments. **B.** Plots of the ratio of acceptor/donor fluorescence intensity versus applied thioredoxin concentrations. The purified and pre-oxidized Redoxfluor protein was incubated with the designated concentrations of thioredoxin, either intact or pre-treated with iodoacetamide (IAA) at 30°C for 2h. The reaction with the intact thioredoxin was also performed with 2.5 mM reduced and 0.25 mM oxidized glutathione in the mixture. The error bars indicate standard deviations. **C.** Modification of Redoxfluor with mPEG-maleimide after the application of thioredoxin. The reaction mixture used in **B** was incubated with the thiol-modifying reagent mPEG-maleimide and subjected to immunoblot analysis for the detection of Redoxfluor. The positions of molecular weight markers (MW) are also shown. The asterisks show band positions of Redoxfluor modified with mPEG-maleimide.

FIGURE 2. Visualization of fluorescence intensity ratio of Redoxfluor expressed in the yeast cytoplasm. **A.** The indicated yeast strain expressing Redoxfluor was cultured in YPD medium to either exponential (EXP) or stationary (STA) phase, and subjected to fluorescence microscopy. The ratio value (FI ratio) reflecting the FRET efficiency of Redoxfluor in the cytosol was calculated from for each point as described in Materials and Methods, and visualized with the designated color gradation. (Note that ratios in the vacuoles were not included in the calculation.) WT, wild type. Bar, 10 μ m. **B.** Comparison of the average FI ratios gained and averaged from 90 cells (1 point per cell). The error bars indicate standard errors of the mean value.

FIGURE 3. Thioredoxin contributions to maintenance of redox status. *A.* The stationary phase strains used in Fig. 2 were kept in the same medium or transferred to water, and incubated at either 30°C or 37°C (mild heat stress) for 4 days. Then the microscopic analysis was done as described in Fig. 2A. Bar, 10 μ m. *B.* The averaged FI ratios were shown as in Fig. 2B. The error bars show standard errors of the mean. *C.* The cells after 4-day incubation in water at 37°C were retrieved and subjected to determination of glutathione levels. The relative amounts of reduced (GSH) and oxidized (GSSG) glutathione were compared among the denoted strains. The error bar indicates standard deviations. The differences in the redox potential of glutathione (ΔE_{GSH}) between wild-type and the mutant and sample were also shown below the graph, along with their standard errors in parenthesis. The positive values indicate more oxidized state of glutathione in the denoted mutant strains. Note that the redox potential of glutathione depends on $[\text{GSH}]^2/[\text{GSSG}]$, not $[\text{GSH}]/[\text{GSSG}]$, and hence the decrease in GSH concentration gives rise to oxidized redox potential even when the ratio ($[\text{GSH}]/[\text{GSSG}]$) is unchanged.

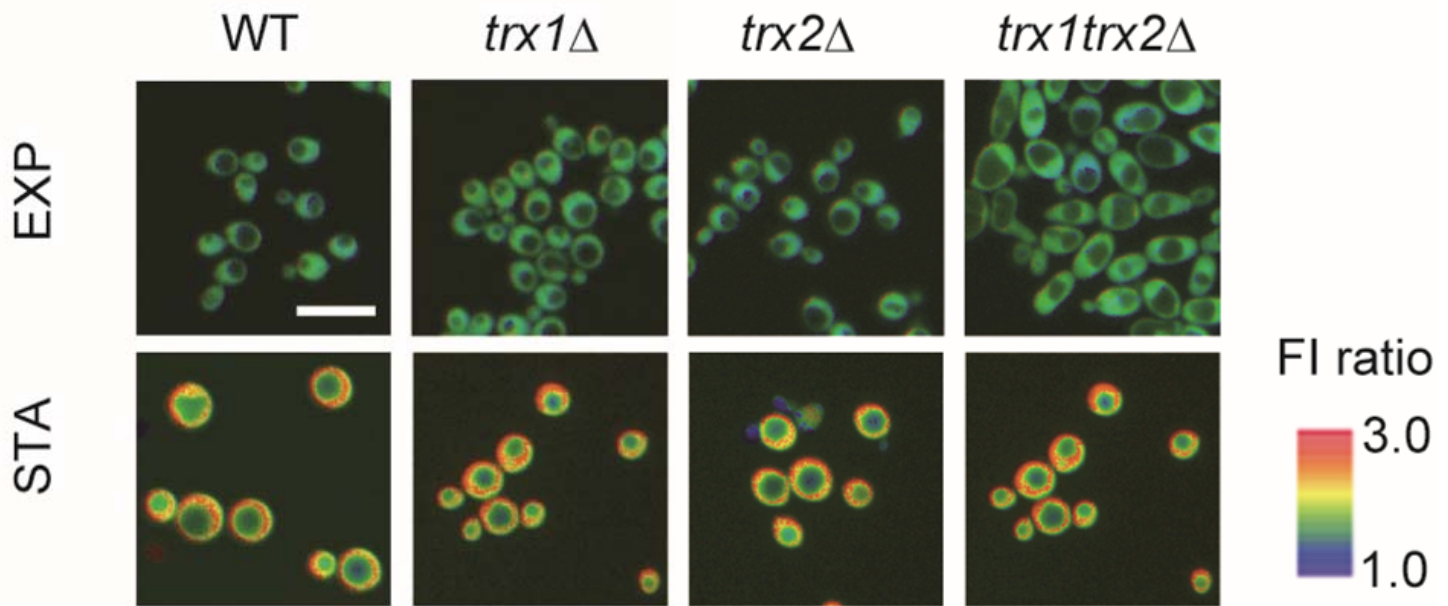
FIGURE 4. TRX2 is required for maintenance of viability during incubation in water at 37°C. The numbers of viable cells for the designated strains after the transfer to water and incubation at 37°C were plotted. The error bars show standard deviations.

Table I *S. cerevisiae* strains^a used in this study

Strain Name	Genotype
YMO300	<i>MATalpha ADE2 can1-100 his3-11 leu2-3,112 trp1-1,15, URA3::pRFY-1A</i> (<i>P_{TDH3}</i> -Redoxfluor- <i>T_{TDH3}</i> in pRS306)
YMO301	YMO300, <i>HIS3:: pRS303; LEU2::pRS305; TRP1::pRS304</i>
YMO302	YMO300, <i>trx1Δ:: (Cg)TRP1; HIS3:: pRS303; LEU2::pRS305</i>
YMO303	YMO300, <i>trx2Δ:: SpHIS5</i> (equivalent to <i>ScHIS3</i>); <i>LEU2::pRS305; TRP1::pRS304</i>
YMO304	YMO300, <i>trx1Δ:: (Cg)TRP1; trx2Δ::SpHIS5; LEU2::pRS305</i>

^a All of the listed strains were generated in this study.

A



B

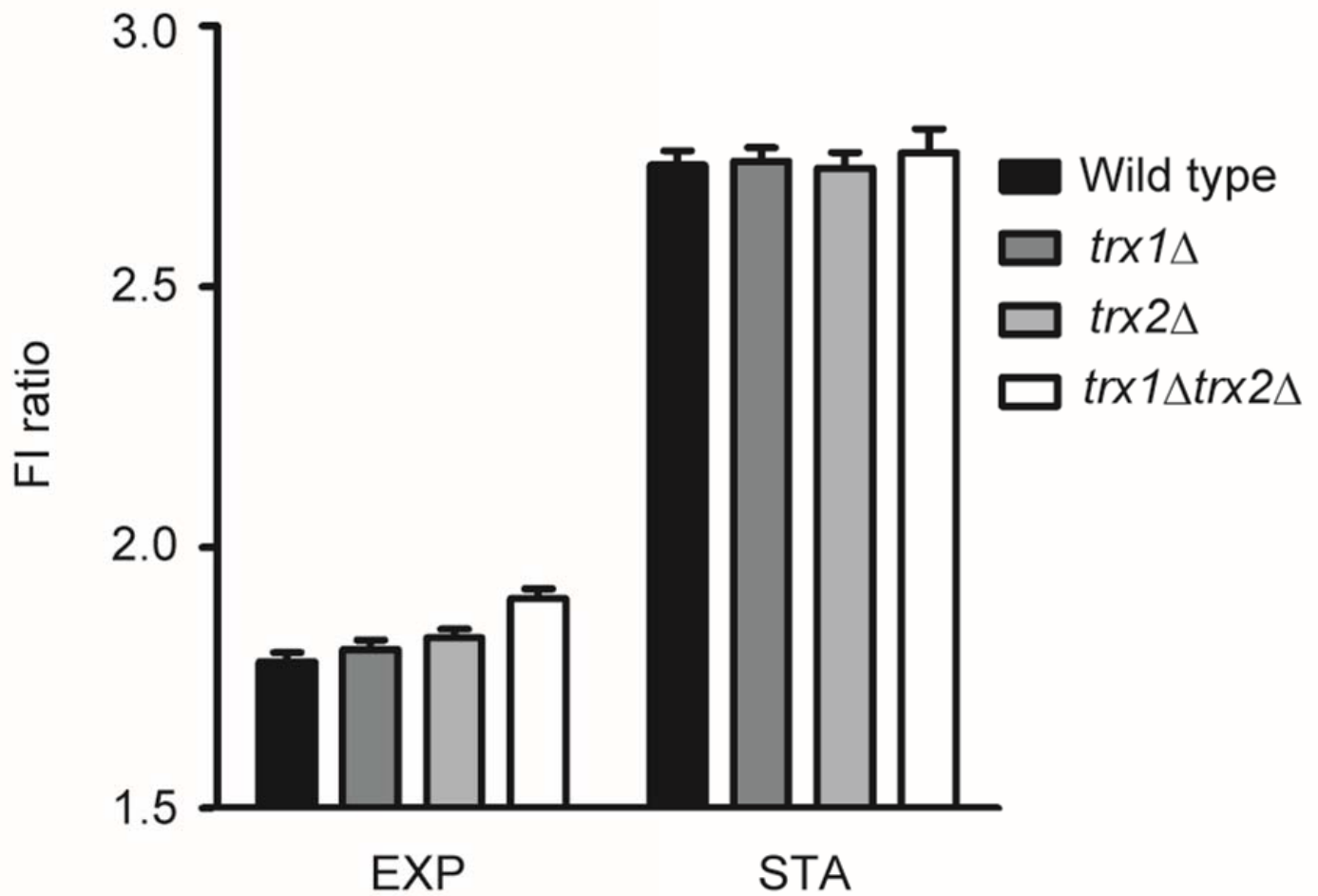


Fig.2 Oku et al

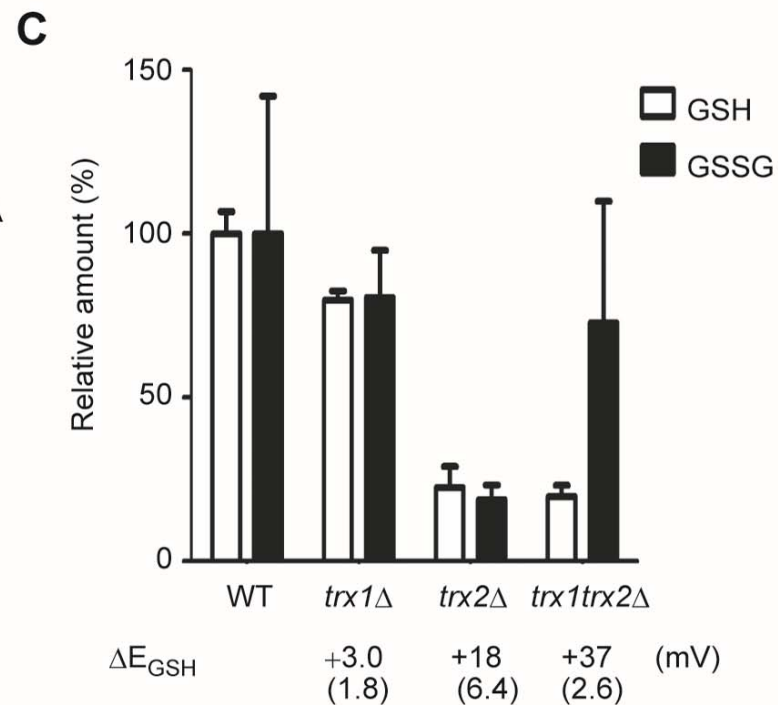
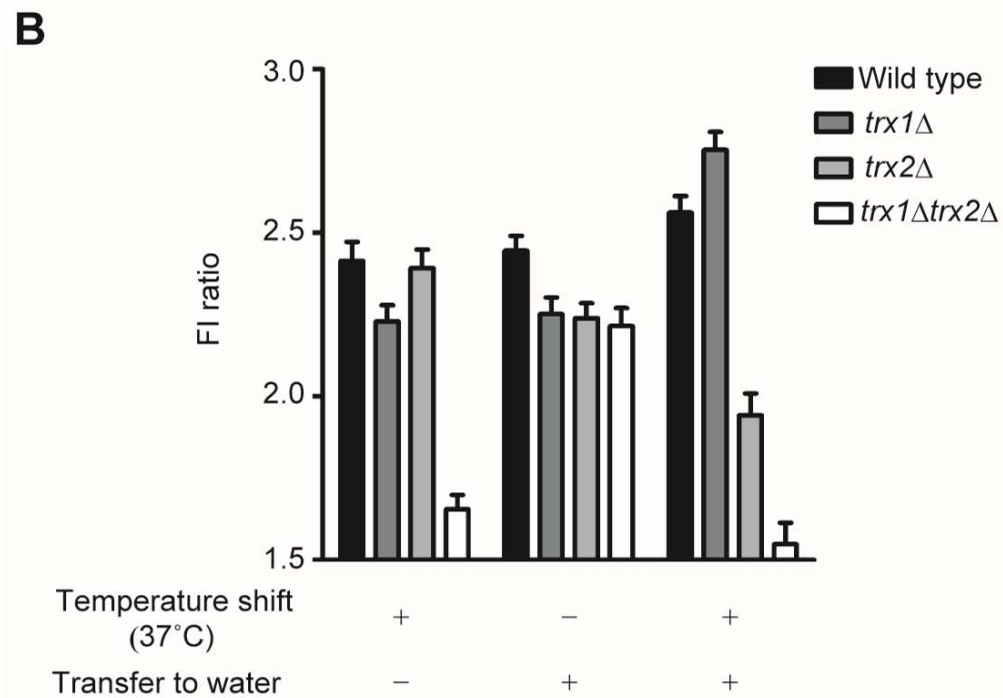
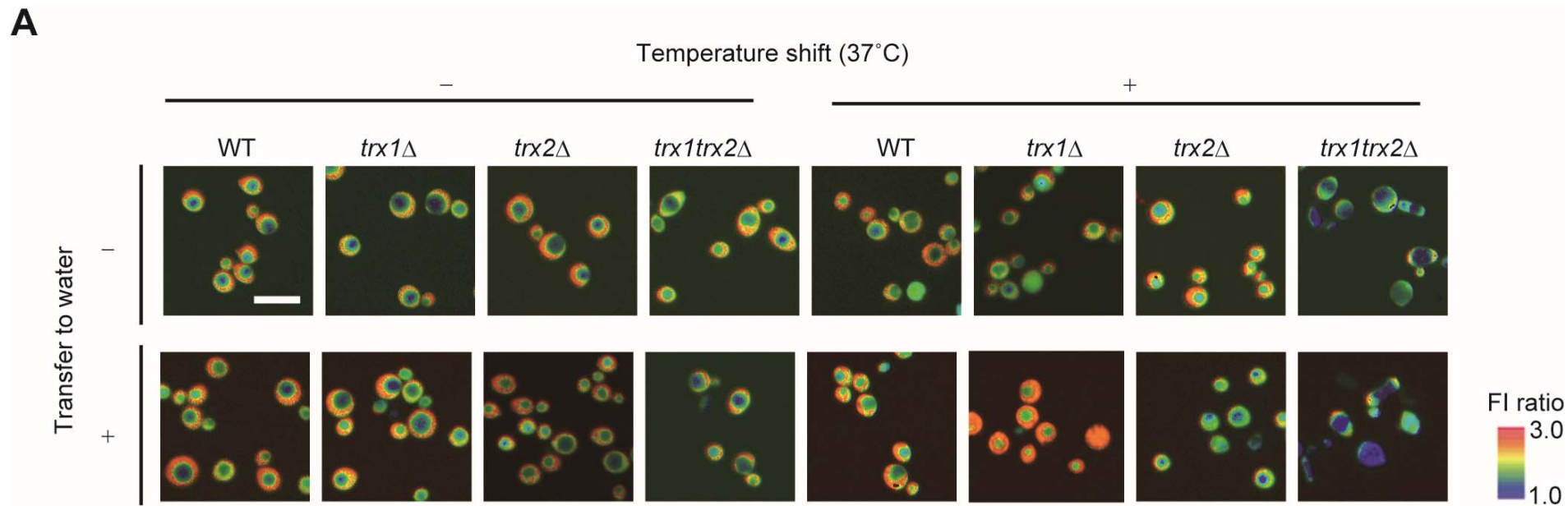


Fig. 3 Oku et al

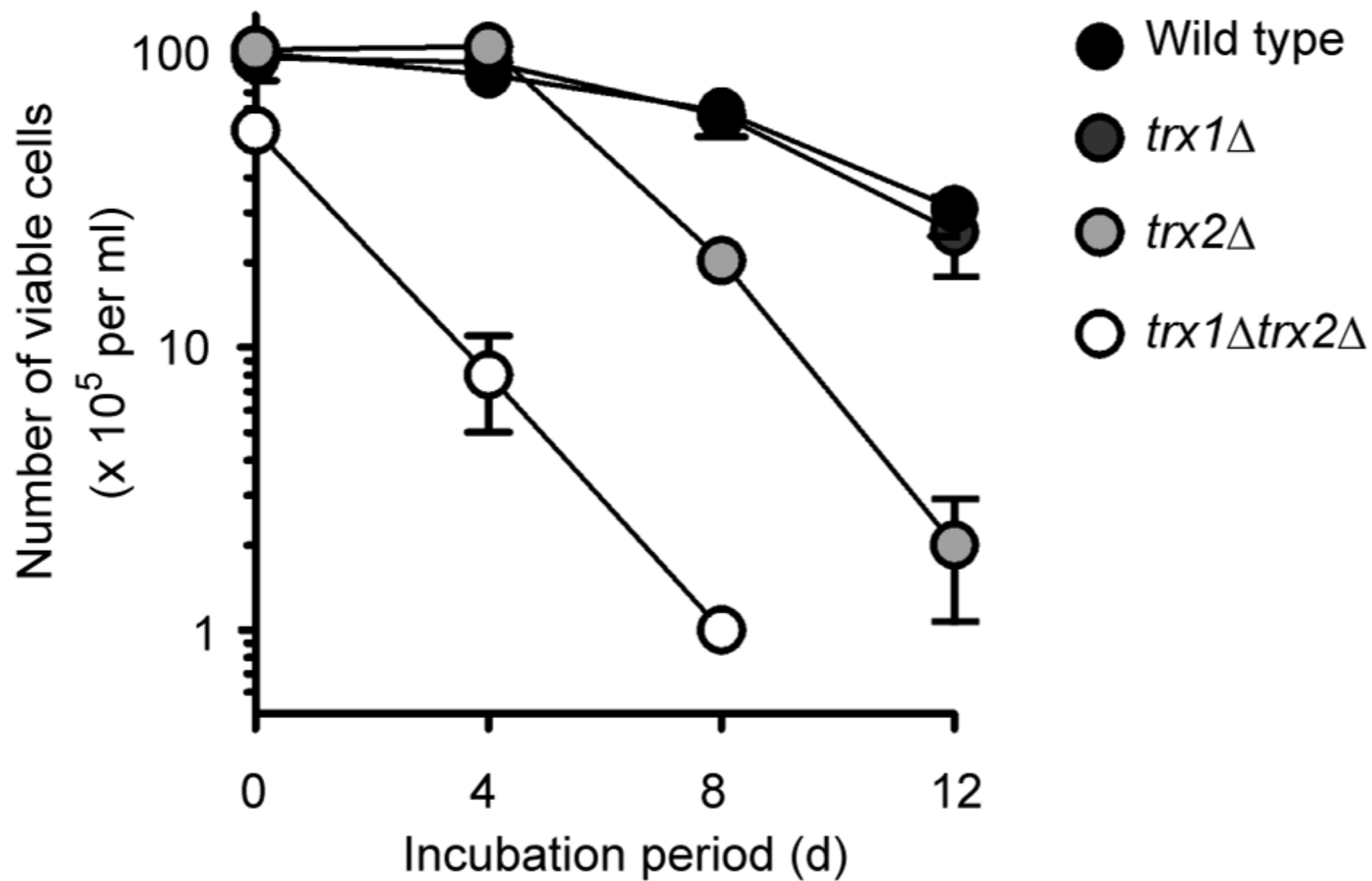


Fig. 4 Oku et al

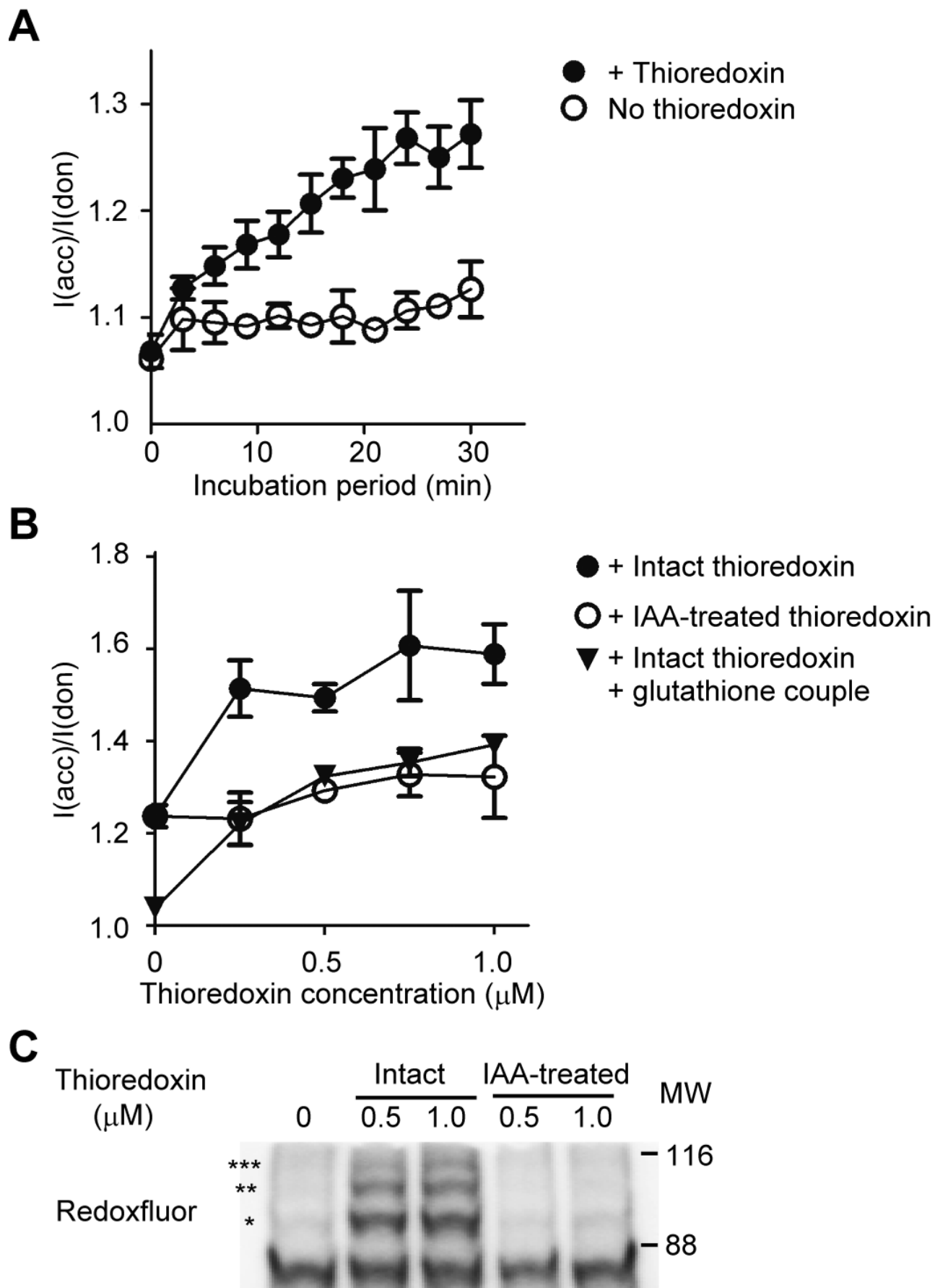


Fig. 1 Oku et al